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Abstract

Aims: Pathological heterogeneity within patients with Frontotemporal lobar degeneration (FTLD) in general precludes the accurate assignment of diagnostic subtype in life. The aim of this study was to assess the extent of microglial cell activation in FTLD in order to determine whether it might be possible to employ this as a diagnostic marker *in vivo* using PET ligand [11C](R)-PK11195 in order to differentiate cases of FTLD according to histological subtype.

Methods: The distribution and extent of microglial cell activation was assessed semi-quantitatively in cortical grey and subcortical white matter of CD68 immunostained sections of frontal and temporal cortex from 78 pathologically confirmed cases of FTLD, 13 of Alzheimer's disease (AD) and 13 controls.

Results: Significantly higher levels of microglial cell activation than controls occurred in all 4 regions in FTLD, and in 3 of the 4 regions in AD. Microglial activation was greater in frontal subcortical white matter in FTLD than AD, whereas it was higher in temporal cortical grey matter in AD than FTLD. Microglial cell activation was significantly higher in temporal subcortical white matter in FTLD-*MAPT* than in other genetic (*GRN*, *C9ORF72*) or non-genetic forms of FTLD.

Conclusions: The present study suggests that high levels of microglial cell involvement in temporal lobe (subcortical white matter) might serve as a marker of inherited FTLD associated with intronic mutations in *MAPT*, with a relatively intense signal in this region in PET studies using [11C](R)-PK11195 as microglial cell marker could indicate the presence of *MAPT* mutation *in vivo*.

Introduction

After Alzheimer's disease (AD), Frontotemporal Lobar Degeneration (FTLD) is the second most common cause of dementia in the under 65s, accounting for 20% of cases [1]. It is a primary neurodegenerative disorder characterized by circumscribed neurodegeneration of the frontal and anterior temporal lobes. FTLD is clinically, pathologically and genetically, heterogeneous, and may in fact represent several distinct disorders united under this umbrella [1].

In pathological terms, about half of cases can be defined by the presence of tau-immunoreactive changes in neurones, and sometimes glial cells, of the frontal and temporal cortex, and hippocampus, characterised by either neurofibrillary tangle-like structures or Pick bodies [2,3]; such cases have been designated as FTLD-tau [4], and some are associated with mutations in the tau gene, *MAPT* [5]. Most of the remaining cases of FTLD are associated with varying combinations of inclusion bodies (neuronal cytoplasmic inclusions (NCI), dystrophic neurites (DN) and neuronal intranuclear inclusions (NII) within neurones of the same regions of cerebral cortex and hippocampus [2,3]. These structures contain the transactive response (TAR) DNA binding protein with Mw 43kDa, TDP-43, [6,7], and such cases are termed FTLD-TDP [4]. Within the FTLD-TDP classification, four histological subtypes are recognised, and termed Types A to D according to the relative proportions of NCI, DN and NII [4]. About half of FTLD-TDP cases are associated with mutations in various genes, but chiefly as insertions/deletions in progranulin gene (*GRN*) [8,9], and most recently in *C9ORF72* gene [10-12], where an expansion of a hexanucleotide repeat within the first intron has now been identified as responsible for disease [10,11]. Lastly, around 5% of cases are characterized by NCI containing fused in sarcoma protein (FUS) [13], and are termed FTLD-FUS [4].

While to some extent there are some close clinical and pathological correlations (eg Semantic dementia (SD) and FTLD-TDP, type C, behavioural variant FTD (bvFTD) and Motor Neurone Disease (FTD+MND) and FTLD-TDP, type B) (see Neary et al [1]), for the most part, and especially so with respect to patients with bvFTD) it is not possible to predict the underlying histology from clinical presentation alone. Genetic analysis can help as mutations in *GRN* and *C9ORF72* predict FTLD-TDP histology [4,9-12], with additional p62 immunoreactive changes within the cerebellum and hippocampus being characteristic of cases with *C9ORF72* expansions [14-16]. *MAPT* mutations are always associated with an underlying tauopathy [5], though this can adopt differing histological appearances, either Pick body-like [17] or neurofibrillary tangle-like [18].

In order to develop rational therapeutic strategies for FTLD that seek to address or correct the underlying histological changes, it will obviously be necessary to be able to predict whether a case is FTLD-tau, FTLD-TDP or FTLD-FUS. As mentioned above, consistent clinicopathological correlations in SD and FTD+MND [1,19] would direct a TDP-43 based therapy, as would genetic changes in *GRN* and *C9ORF72* [8-12]. However, such a rationale would not be applicable for most cases of bvFTD where an underlying tau, TDP-43 or FUS histology is possible [4], and if no identifiable gene is present, such cases could not be confidently differentiated on clinical assessment alone. A blood or cerebrospinal fluid based biomarker might be useful, though currently no such validated marker is available. PET imaging has been used to identify early cases of AD based on PIB binding to beta-amyloid plaques [20], and although PET markers that might have the potential to indicate the presence of tau pathology are currently being evaluated [21], there are no known equivalent pathology-based radioligands that could definitely identify the presence of, TDP-43 or FUS histology.

Over the last 10 years, it has become possible, using the PET ligand [11C](R)-PK11195, to image microglial activation, and this seems to correspond well to the distribution of the underlying pathological changes in Parkinson's disease and AD [22,23]. Small pilot studies have also been successful in showing the presence of microglial activation *in vivo* in progressive supranuclear palsy (PSP) and cortical basal degeneration (CBD) [24,25], and also in a small cohort of FTLD patients [26]. However, no correlative genetic or neuropathological data was available for any of these studies.

Given its heterogeneous pathology, it is possible that the different types of FTLD (ie FTLD-tau, FTLD-TDP and FTLD-FUS) might show different patterns of microglial cell activation depending on the underlying pathology. Should this be the case a modality like [11C](R)-PK11195 PET could clearly be helpful in differentiating FTLD subtypes *in vivo*. With this in mind, we sought to investigate patterns of microglial cell activation across the different clinical, histological and genetic forms of the disorder in an attempt to ascertain whether any one histological form of FTLD, especially those associated with bvFTD, might be differentiated according to the extent of this pathological change.

Patients and Methods

Patients

One hundred and four cases were investigated. All were obtained from the Manchester Brain Bank through appropriate consenting procedures for the collection and use of the human brain tissues. The patient groups comprised 78 patients with FTLD, 13 with AD and 13 control subjects (see on-line supplementary Table 1). The mean age of disease onset in the FTLD group was 60.0±8.6 years (range 43-73 years), the mean duration of disease was 7.6±3.7 years (range 2-18 years) and the mean age at death was 67.7±8.1 years (range 45-77 years). The mean age of disease onset in the AD group was 64.4±12.2 years (range 48-79 years), the mean duration of disease was 10.0±3.7 years (range 5-18 years) and the mean age at death was 74.9±11.2 years (range 57-87 years). In the control group, mean age at death was 52.9±20.9 years (range 26-87 years).

All FTLD cases fulfilled the original Lund-Manchester clinical diagnostic criteria for FTLD [27,28], and were consistent with recent consensus criteria [29,30]. All had been longitudinally assessed within the Cerebral Function Unit, Salford Royal Hospital, employing the Manchester Neuropsychological Test Battery. Data collected on each patient included gender, age at diagnosis and at death, and clinical diagnosis. All cases were from the Manchester Brain Bank. Pathological diagnoses were made by an experienced Neuropathologist (Professor David Mann) and genetic analyses (for *C9ORF72*, *MAPT* and *PGRN*) were performed in the laboratory of Professor Stuart Pickering-Brown (see [5,8,11] for methodological details).

The FTLD group was composed of 46 males and 32 females (see on-line Supplementatry Table 1). Forty three patients had been clinically diagnosed with bvFTD, 9 with FTD+MND, 7 with SD, 8 with progressive non-fluent aphasia (PNFA), 9 with PSP/CBD, 1 with Multiple System Atrophy (MSA) and 1 with MND. According to the pathological classification of Mackenzie et al [4], 44 patients had FTLD-TDP, of which 19 had type A pathology, 18 had type B pathology and 7 had type C pathology. Thirty four cases had FTLD-tau, of which 8 had the histology of Pick's disease, 10 had tau pathology consistent with possession of exon 10 +16 *MAPT* mutation and 16 had CBD/PSP pathology.

Twenty seven of the FTLD cases bore a mutation on the *MAPT*, *GRN* or *C9ORF72* gene. Of these, 8 bore a mutation on *GRN* gene, 1 with exon 1 C31LfsX34, 1 with exon 4 Q130SfsX124, 4 with exon 10 V452WfsX38 mutation, 1 with exon 10 Q468X, and 1 with exon 11 R493X. These all had FTLD-TDP type A pathology, 4 with bvFTD and 2 with PNFA. 10 patients bore *MAPT* mutation (9 with +16 splice site mutation on intron to exon 10 and 1 with +13 splice site mutation on intron to exon 10). The other 9 patients bore a hexanucleotide repeat expansion on *C9ORF72* gene. Four of these had FTLD-TDP type A based pathology, with a diagnosis of bvFTD, and 5 had a pathological diagnosis of FTLD-TDP type B, 3 with FTD+MND and 2 with bvFTD. The mean duration of disease for each mutation type was similar (8-10 years), though the mean age at onset of disease varied significantly (60.5±5.1 years for *GRN*; 51.6±5.7 years for *MAPT* and 59.6±6.3 years for *C9ORF72*; p=0.028 between *GRN* and *MAPT*).

The 13 controls (6 males and 7 females) were judged to be clinically normal and none showed any pathology beyond that which might be anticipated for age. Three cases (over 65 years of age) showed mild deposition of amyloid beta protein, mostly in the form of diffuse amyloid plaques, one of which showed a mild neurofibrillary pathology confined essentially to the amygdala and hippocampal formation. Two other cases showed moderate cerebrovascular disease: the remaining 8 cases were apparently histologically normal. The 13 patients with AD all met pathological criteria for definite AD [31,32], and were Braak stage V or VI.

Methods

Immunohistochemistry

Sections of frontal (Brodmann areas 8/9) and temporal cortex (Brodmann areas 21/22) were cut at 6μm thickness from formalin fixed, paraffin embedded blocks and mounted on to glass slides. Sections were firstly hydrated through successive baths of xylene, alcohols of decreasing concentration and distilled water. Antigen unmasking was performed by pressure cooking in citrate buffer (pH 6 10mM) for 30 minutes, reaching 120 degrees Celsius and >15 kPa pressure. Sections were incubated for 30 minutes at room temperature in 0.3% peroxide in methanol to quench endogenous peroxidise activity, and then for a further 30 minutes at room temperature in Vectastain Elite PK-6101 goat serum as blocking buffer. Sections were then incubated for one hour at room temperature in mouse monoclonal antibody CD68 (Dako Cytomation, Glostrup, Denmark, DK-2600) at a concentration of 1:100. The sections were incubated for 30 minutes in a biotinylated secondary antibody followed by 30 minutes in Avidin Biotin Complex (ABC) reagent (both Vectastain Elite PK-6101 Rabbit IgG), both at room temperature. Sites of immunoreactions were visualised by incubating in DAB (3,3′-diaminobenzidine tetrahydrochloride) for 5 minutes, followed by light counterstaining with haematoxylin (Vector H-3401). Sections were dehydrated and mounted for analysis under the microscope.

Microscopic analysis

The CD68 antibody immunostains microglial cells, both in a resting (r) and activated (a) condition (Figure 1). Sections of frontal and temporal cortex were assessed for the presence of immunostained microglial cells within both the cortical grey and subcortical white matter at x20 magnification.

The frequency and ‘severity’ (in terms of morphological types, with activated microglial cells being considered to be more ‘severe than ramified microglial cells) of CD68-immunostained sections was assessed according to:

0 = no immunostained cells present.

1 = very few immunostained cells present, all as ramified microglia.

2 = a moderate number of immunostained cells present, mostly ramified but some activated cells present.

3 = many, diffusely spread, immunostained cells present, all as activated microglia.

4 = many large clusters of activated microglial cells present.

All assessments were made by a single observer (SBL), blinded to diagnosis. Sections were scored twice to increase objectivity, and any discrepancies reconciled by consultation with a second observer (DMAM). In addition a random set of 8 sections were scored on a weekly basis over the course of the study.

Statistical Analysis

Rating data was entered into an excel spreadsheet and analysed using Statistical Package for Social Sciences (SPSS) software (version 17.0). A p-value of less than 0.05 was considered statistically significant. The data was subjected to testing for homogeneity, skewness and kurtosis. If any of these results were significant, the data was considered non-parametric and Kruskal-Wallis test was performed when 3 or more groups were analysed. If this detected any significant differences between the groups, post-hoc testing was performed to identify the groups between which significant differences existed. Mann-Whitney test was used to compare rating data between pairs of groups.

Results

Histological appearances

Both the phenotype and physiological function of microglial cells are highly plastic, relating to their level of activity. As such, microglial morphology depends upon the type and degree of pathology present, and on signalling from other cellular structures, such as surrounding neurons and astrocytes. A 'ramified' microglial cell phenotype, characterised morphologically by a thin cell body with long, branching processes (Figure 1 - r) is seen when no apparent tissue pathology is present. Following Toll-like receptor-mediated recognition of endogenous damage-associated molecular patterns (DAMPs) or non-self pathogen-associated molecular patterns (PAMPs), microglial cells condense from a quiescent state of surveillance into a macrophage-like phagocytic phenotype (Figure 1 - a). In general, the topographic distribution of activated microglial cells followed that of the principal pathological changes present within the frontal and temporal cortex (ie amyloid and tau pathology), whether this was in FTLD or AD, or in the control cases.

In FTLD-TDP type A and type C cases, generally, there were more activated microglial cells in the upper (I-III) cortical layers than in the lower (IV-VI) cortical layers, and more so in the subcortical white matter than in the cortical grey matter. However, in FTLD-TDP type B cases, there was a more uniform distribution of microglial cells across the laminae of the cortical grey matter, with comparable levels of activated microglial cells in cortical grey and subcortical white matter.

In FTLD-tau associated with Pick bodies, there was a fairly uniform distribution of microglial cells across all laminae of the cortical grey matter, but these were generally more frequent in cortical grey (Figure 2a), than subcortical white (Figure 2b), matter, especially in areas where Pick bodies were dense (Figure 2c). However, in FTLD-tau associated with *MAPT* mutation, there were many more microglial cells in subcortical white (Figure 2d), than cortical grey (Figure 2e), matter, in line with the greater density of tau pathology in oligodendroglial cells in subcortical white matter (Figure 2f). In PSP/CBD, clustered microglial cells appeared less frequent in cortical grey (Figure 3a) than subcortical white (Figure 3b) matter, despite a high density of both astrocytic plaques in cortical grey matter (Figure 3c) and coiled bodies in subcortical white matter (Figure 3d).

As previously reported [33], microglial cells were more common in cortical grey matter in AD compared to subcortical white matter, often clustered within and around amyloid plaques (not shown). In the control cases (Figure 1), activated microglial cells were largely absent, though a few were occasionally seen in respect to rare amyloid deposits. Ramified microglial cells were commonly seen in many of these cases.

Semiquantitative analyses

Scores for microglial cell 'severity' in cortical grey and subcortical white matter, of both frontal and temporal cortex, were compared separately across, and within, the different diagnostic groups.

Microglial cell scores were highly significantly different between FTLD (overall), AD and control groups for temporal cortical grey matter ($F_{2,103}$=22.7; p<0.001) and frontal cortical white matter ($F_{2,104}$=17.9; p<0.001), and marginally significant for temporal cortical white matter ($F_{2,103}$=65.6; p=0.059) and frontal cortical grey matter ($F_{2,84}$=6.4; p=0.042). Post hoc testing showed microglial scores were higher in FTLD than controls in all 4 regions (frontal cortical grey matter (p=0.022), temporal cortical grey matter (p=0.022), frontal cortical white matter (p=0.001) temporal cortical white matter (p=0.023)). Microglial scores were significantly higher in AD than controls in both frontal (p=0.019) and temporal (p<0.001) cortical grey matter, and also marginally so in temporal subcortical white matter (p=0.039), but not in frontal subcortical white matter (p=0.880). Moreover, microglial scores were significantly higher in frontal subcortical white matter in FTLD than in AD (p=0.002), but were higher in temporal cortical grey matter in AD than in FTLD (p<0.001). There were no significant differences in scores for AD and FTLD in either frontal cortical grey matter (p=0.609) or in temporal subcortical white matter (p=0.929).

The FTLD cases were stratified according to histological subtype (ie FTLD-tau, FTLD-TDP) and differences in microglial scores were sought between subgroups. Overall, there were no significant differences in microglial cell scores between FTLD-tau and FTLD-TDP (frontal cortical grey matter (p=0.996), temporal cortical grey matter (p=0.226), frontal subcortical white matter (p=0.531) or temporal subcortical white matter (p=0.287)), though if the PSP/CBD cases were excluded, there was significantly higher microglial scores in both temporal cortical grey (p=0.022) and subcortical white (p=0.017) matter in FTLD-tau than FTLD-TDP (p=0.022).

Microglial cell scores differed significantly between FTLD-tau subtypes (Picks vs *MAPT* vs CBD/PSP) in temporal cortical grey ($F_{2,34}$=6.8; p=0.034) and subcortical white ($F_{2,34}$=10.04; p=0.007) matter, but not in frontal cortical grey ($F_{2,34}$=0.84; p=0.656) or subcortical white ($F_{2,34}$=2.4; p=0.296) matter. Post hoc testing for temporal cortex showed that there were significantly higher microglial scores in FTLD-*MAPT* cases than CBD/PSP cases for both cortical grey (p=0.009) and subcortical white (p=0.001) matter, but there were no significant differences between FTLD-*MAPT* and FTLD-Picks (p=0.237 and p=0.101, respectively), or FTLD-Picks and PSP/CBD (p=0.490 and p=0.320, respectively) cases. On the other hand, there were no significant differences in microglial scores between any of the FTLD-TDP histological subtypes (ie type A vs type B vs type C) for any of the 4 regions (frontal cortical grey matter ($F_{2,44}$=2.7; p=0.254, frontal cortical white matter ($F_{2,44}$=4.4; p=0.111), temporal cortical grey matter ($F_{2,43}$=0.25; p=0.882) temporal subcortical white matter ($F_{2,43}$=1.9; p=0.387)).

FTLD cases were also compared according to genetic subtype (ie *GRN* vs *C9ORF72* vs *MAPT* vs no known genetic association). Microglial cell scores again differed significantly between the genetic subtypes in temporal subcortical white matter ($F_{4,61}$=10.0; p=0.040) and marginally so in temporal cortical grey matter ($F_{4,61}$=8.7; p=0.069) matter, but not in frontal cortical grey ($F_{4,62}$=1.07; p=0.898) or subcortical white ($F_{4,62}$=2.8; p=0.598) matter. Post hoc testing showed that microglial scores were significantly higher in *MAPT* mutation cases than in *GRN* (p=0.003) or *C9ORF72* (p=0.054) cases, or in cases with no gene association (p=0.009) (Figure 4).

In the control group, there were no significant correlations between microglial cell scores in either frontal or temporal cortical grey or subcortical white matter and age at death.

Discussion

In the present study we have assessed and compared levels of microglial cell activation in frontal and temporal cortical grey and subcortical white matter regions in cases of FTLD and AD versus control subjects, and within FTLD cases alone have compared differed histological and genetic subtypes. As would be expected, we found higher levels of microglial cell activation than controls in all 4 regions in FTLD, and in 3 of the 4 regions studied in AD. Microglial cell activation was greater in frontal subcortical white matter in FTLD compared to AD, whereas it was higher in temporal cortical grey matter in AD compared to FTLD. Such observations emphasize the importance of neuroinflammation, both microglial and astrocytic in origin, in neurodegenerative disease [34-36].

The patterns of microglial cell activation in FTLD and AD are likely to reflect the distribution of other pathologies, with the well known association between microglial cells and amyloid plaques, especially cored plaques [33], leading to high cortical grey matter levels in AD compared to FTLD and controls, and the intense oligodendoglial tau pathology in subcortical white matter in FTLD cases associated with intronic mutations in exon 10 [18].

When comparing FTLD cases, although levels of microglial cell activation were higher ‘across the board’ than in controls, no significant differences were noted when comparing FTLD-tau with FTLD-TDP-43 groups, or when FTLD-TDP histological (type A vs type B vs type C) or genetic (*GRN* vs *C9ORF72*) subtypes were compared, suggesting that microglial activation is generally upregulated in both tau and TDP-related pathologies. A number of studies have also implied this [37,38].

Nonetheless, it was found that against this general increase in microglial cell activation across all major histological subtypes, the level of microglial cell activation in temporal subcortical white matter in FTLD-*MAPT* cases was significantly higher compared with that in the same region in CBD/PSP cases or in the other genetic forms of FTLD (ie *GRN*, *C9ORF72* or no known mutation), though no differences across these groupings were seen in the other 3 brain regions This finding was perhaps unexpected since it is known that PGRN regulates brain inflammatory responses, and that there is upregulation of PGRN in microglial cells in patients bearing *GRN* mutations [39]. Consequently, it might have been anticipated that cases with *GRN* mutations and TDP type A histology would also have displayed relatively high levels of microglial cell activation compared to other genetic or histological subtypes. However, it has been recently shown that microgliosis only occurs in homozygous *GRN* knock-out mice, and although heterozygotes display a similar clinical phenotype, resembling human bvFTD, this is not associated with any inflammatory brain changes [40]. Hence, heterozygous *GRN* mutations in human FTLD, while directing PGRN haploinsufficiency, may not as shown here be associated with any (excessive) microglial cell activation, and that microgliosis here may derive indirectly from (products of) the neurodegenerative process involving neuronal cell death, rather than via a direct activation of microglial cells through PGRN deficiency.

Nonetheless, the present study suggests that high levels of microglial cell involvement in temporal lobe (subcortical white matter) might serve, at least, as a surrogate for that form of inherited FTLD associated with intronic mutations in *MAPT*. Such correlations between the amount or extent of microglial cell activation in the underlying pathology and genetic predisposition have potential implications for PET studies aimed at imaging microglial cell activation *in vivo* with [11C](R)-PK11195. In this context the relatively intense signal in the subcortical white matter of the temporal lobe (compared to other histological and genetic forms of FTLD) could indicate the presence of a *MAPT* mutation.

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Suzannah Lant did all the immunohistochemistry and microscopical assessments, and helped with paper writing

Andrew Robinson prepared sections for staining and immunohistochemistry

Jennifer Thompson did data analysis

Julie Snowden helped with statistical advice and clinical data

Yvonne Davidson provided technical support and training

Alexander Gerhard helped with discussions and paper writing

David Mann provided study design, supervision, helped with microscopical assessments and wrote the paper

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Legends to Figures

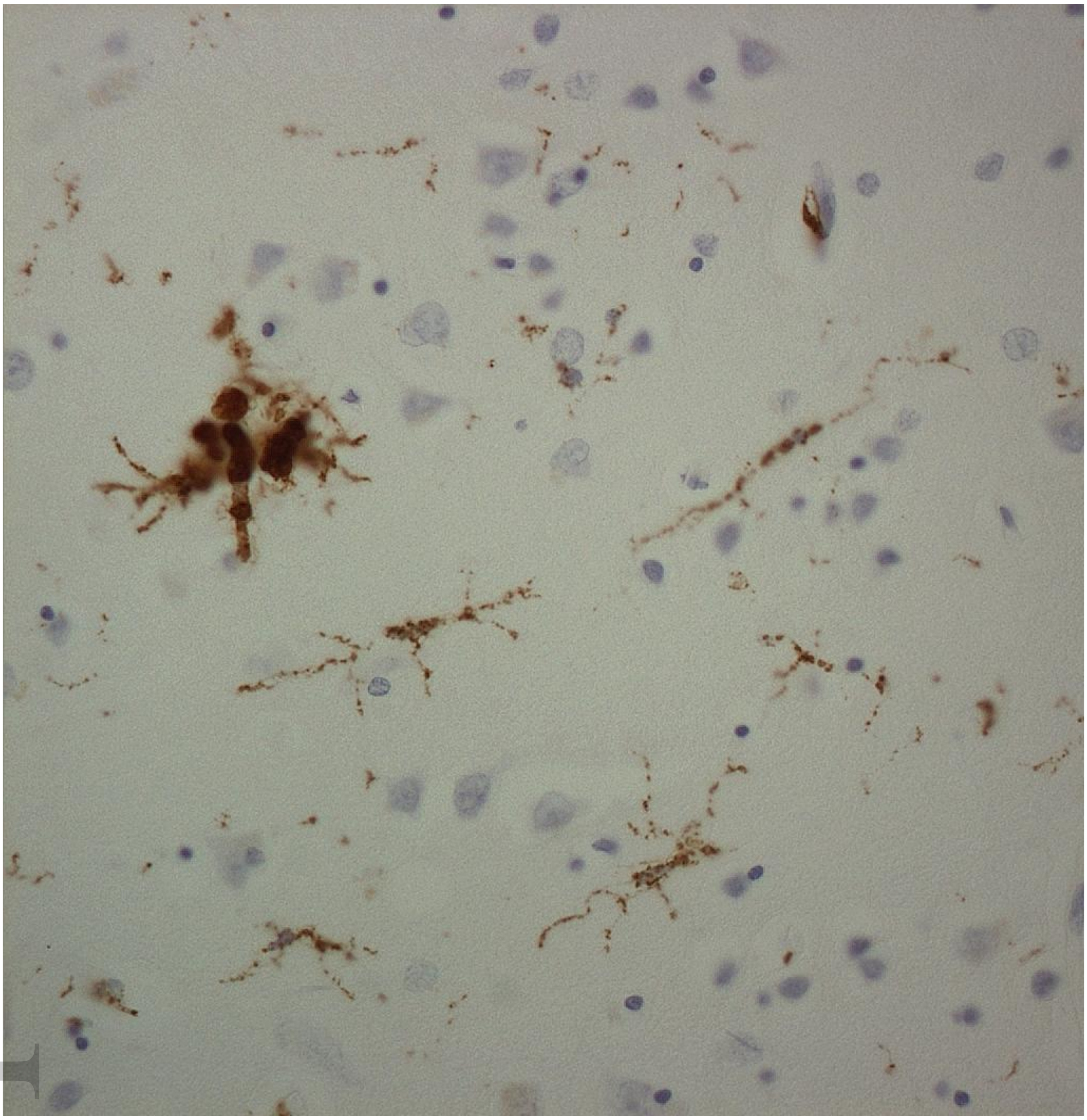
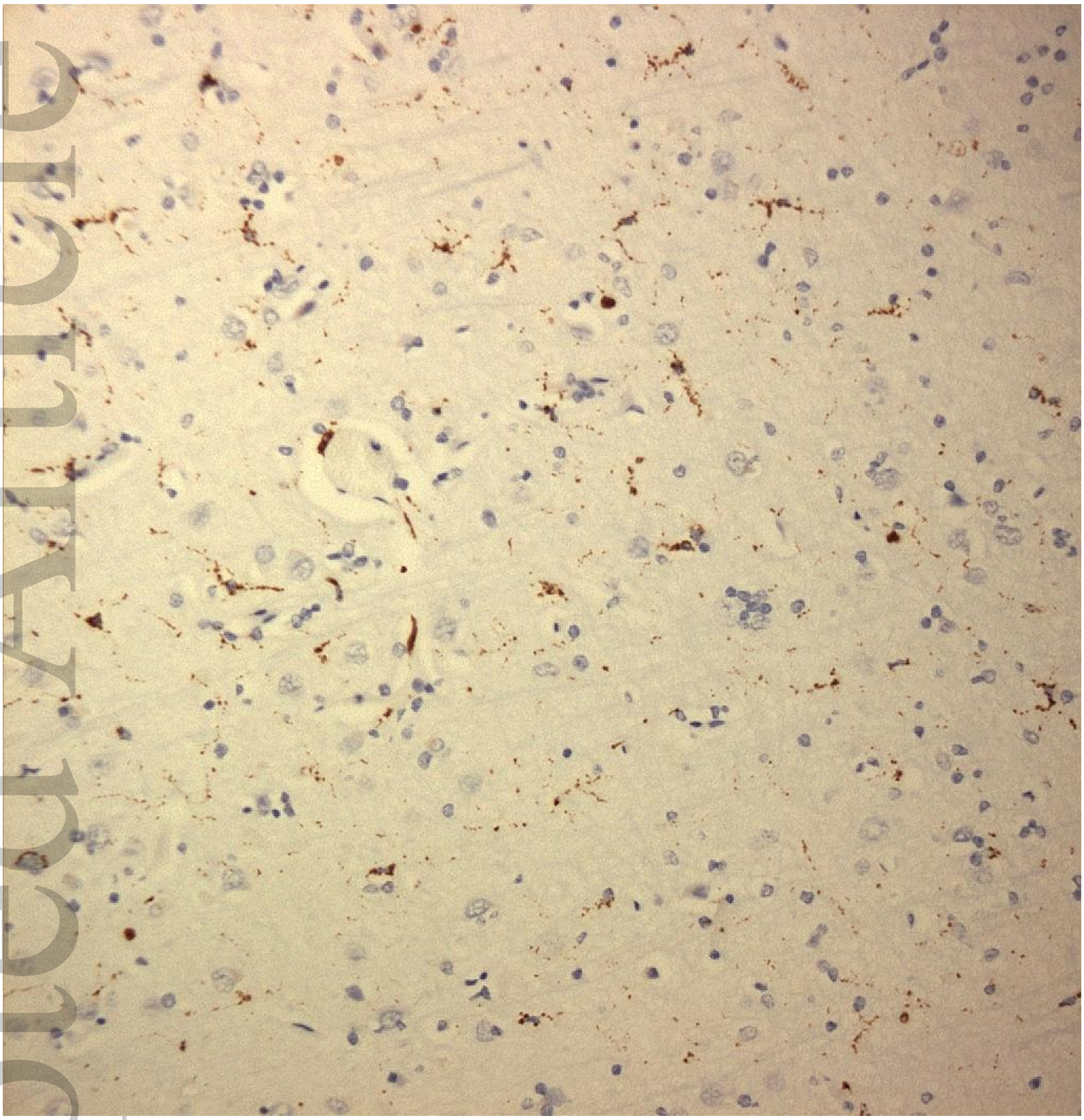
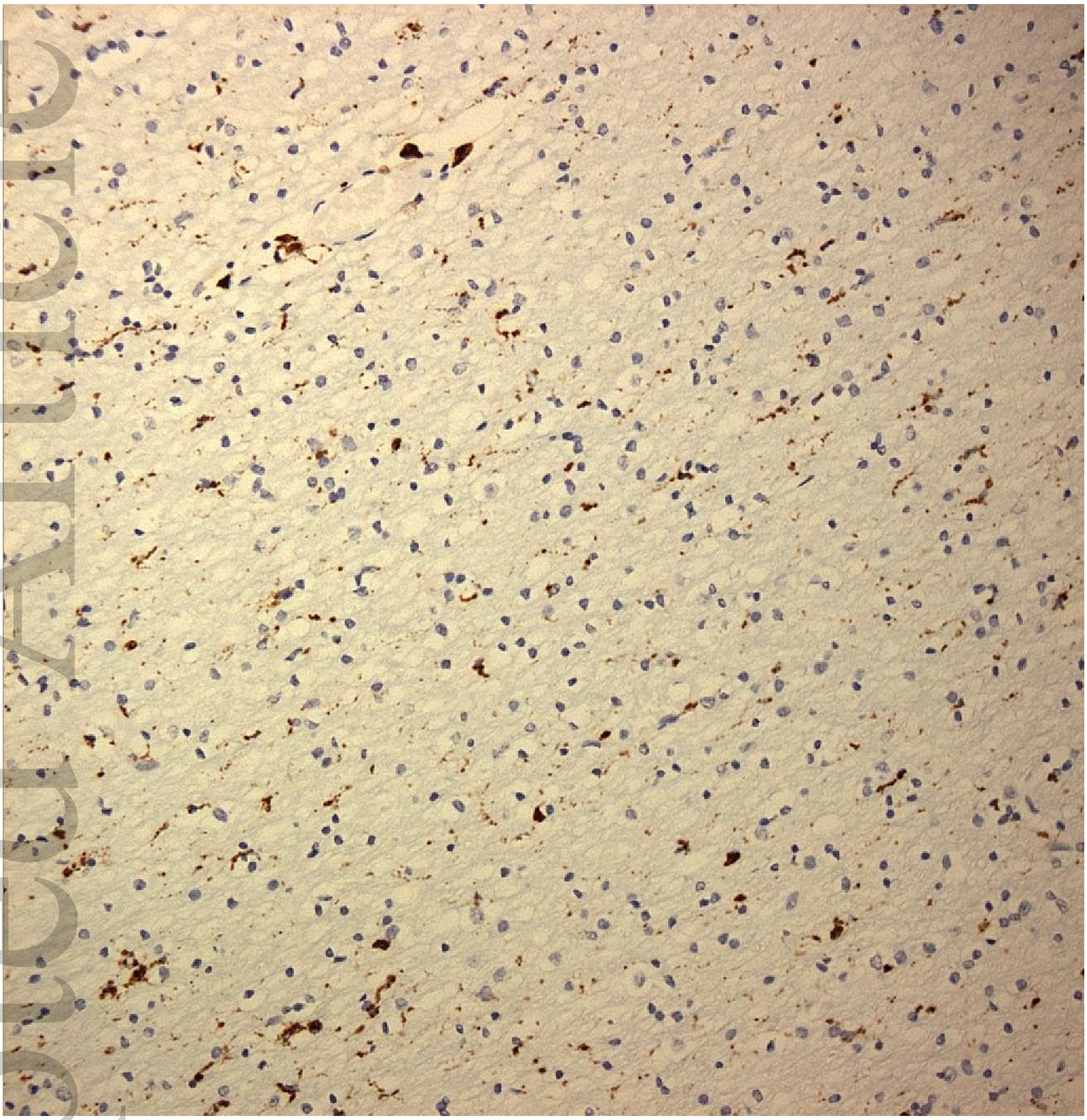
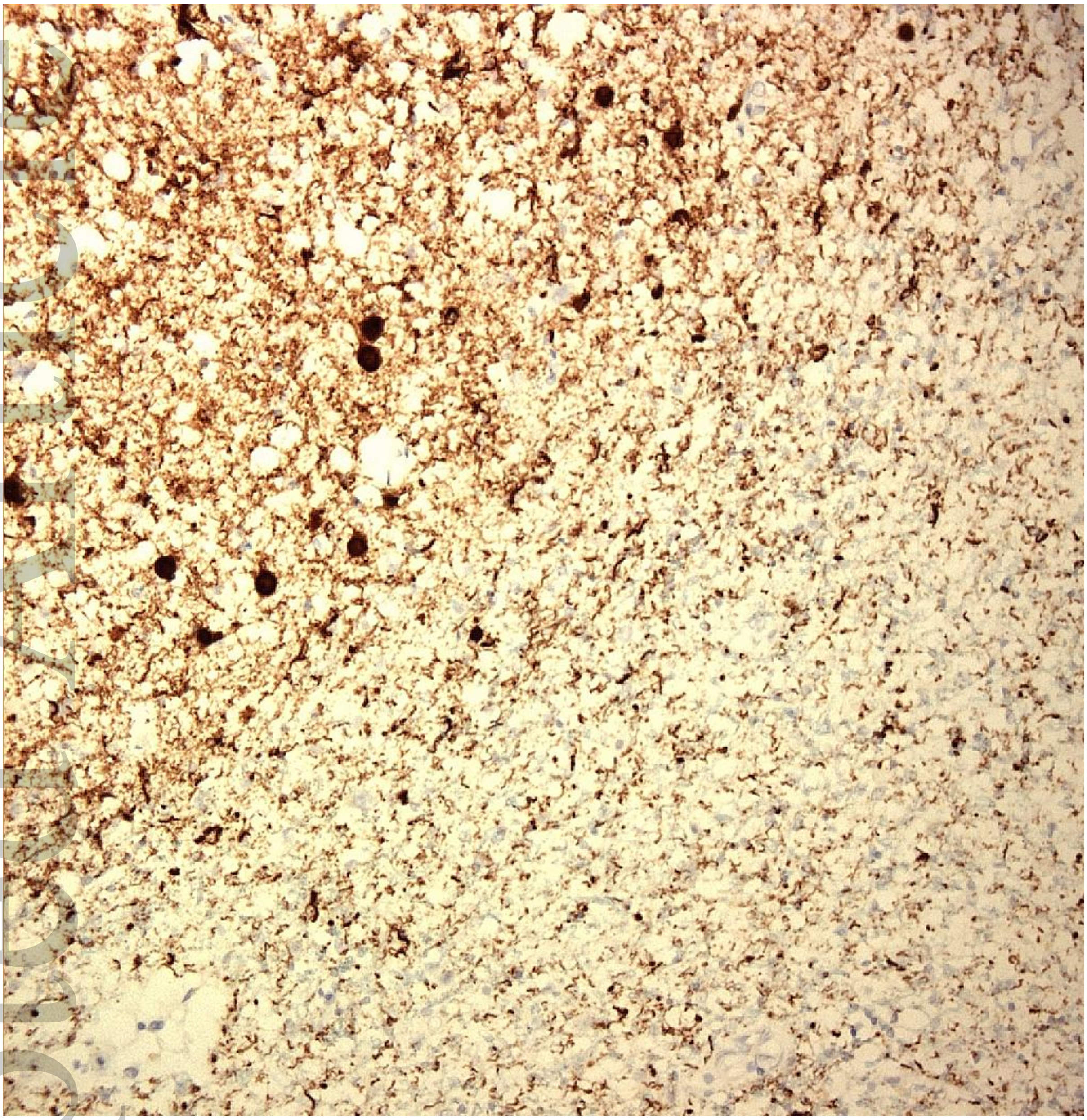
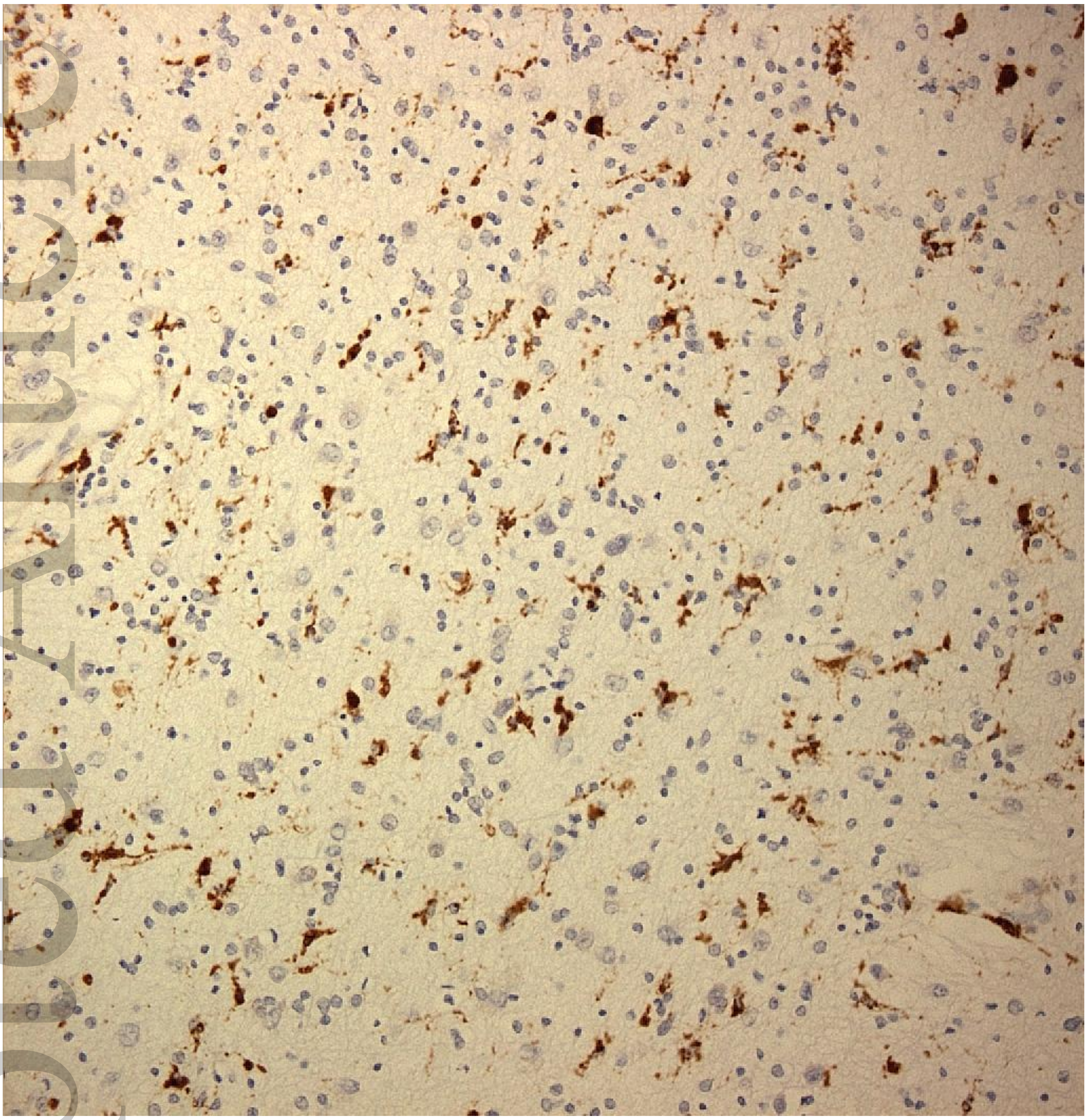


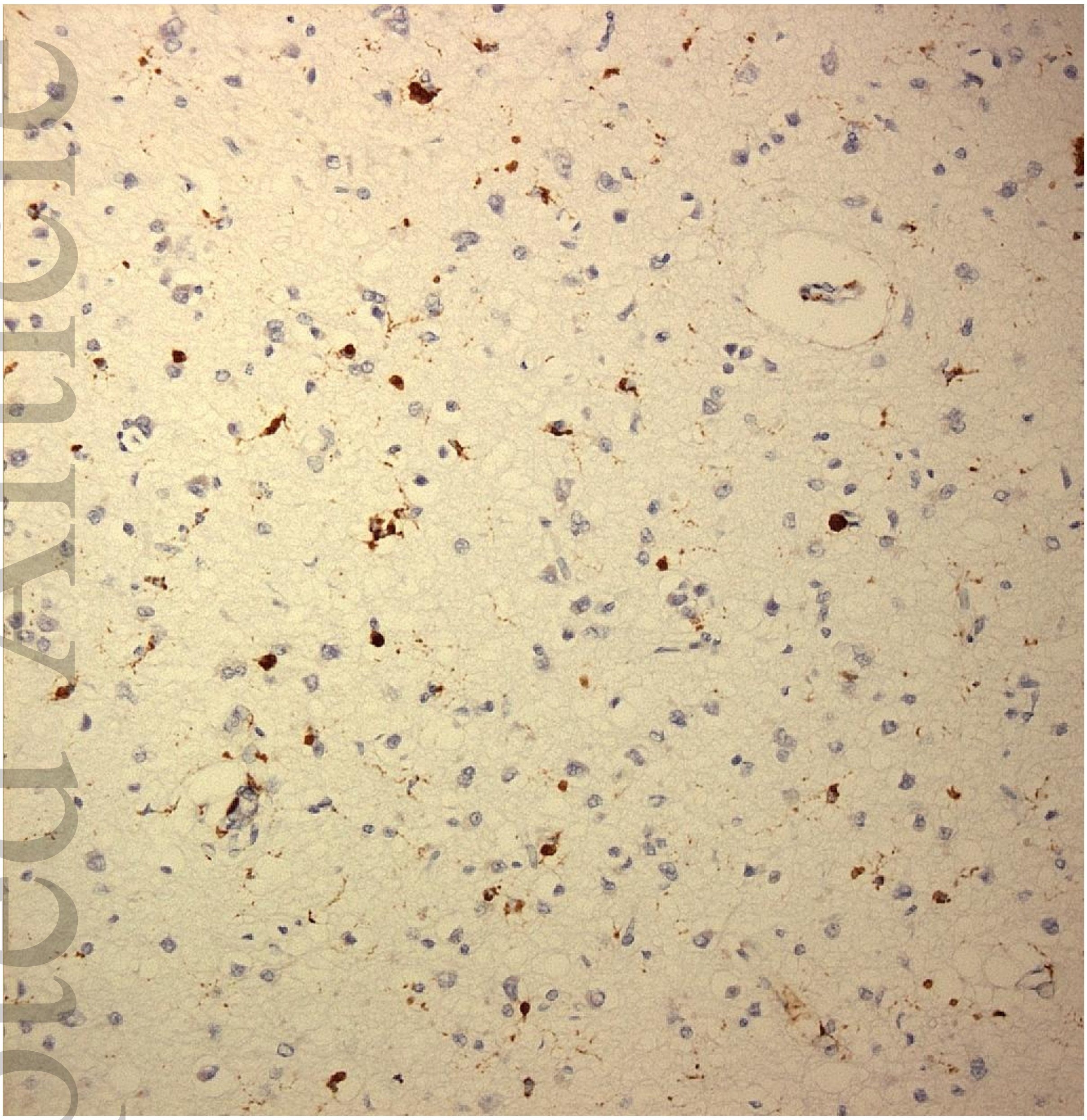
Figure 1. Activated (a) and ramified (r) microglial cells, as seen in CD68 immunostaining. Immunoperoxidase – haematoxylin; x40 microscope objective magnification











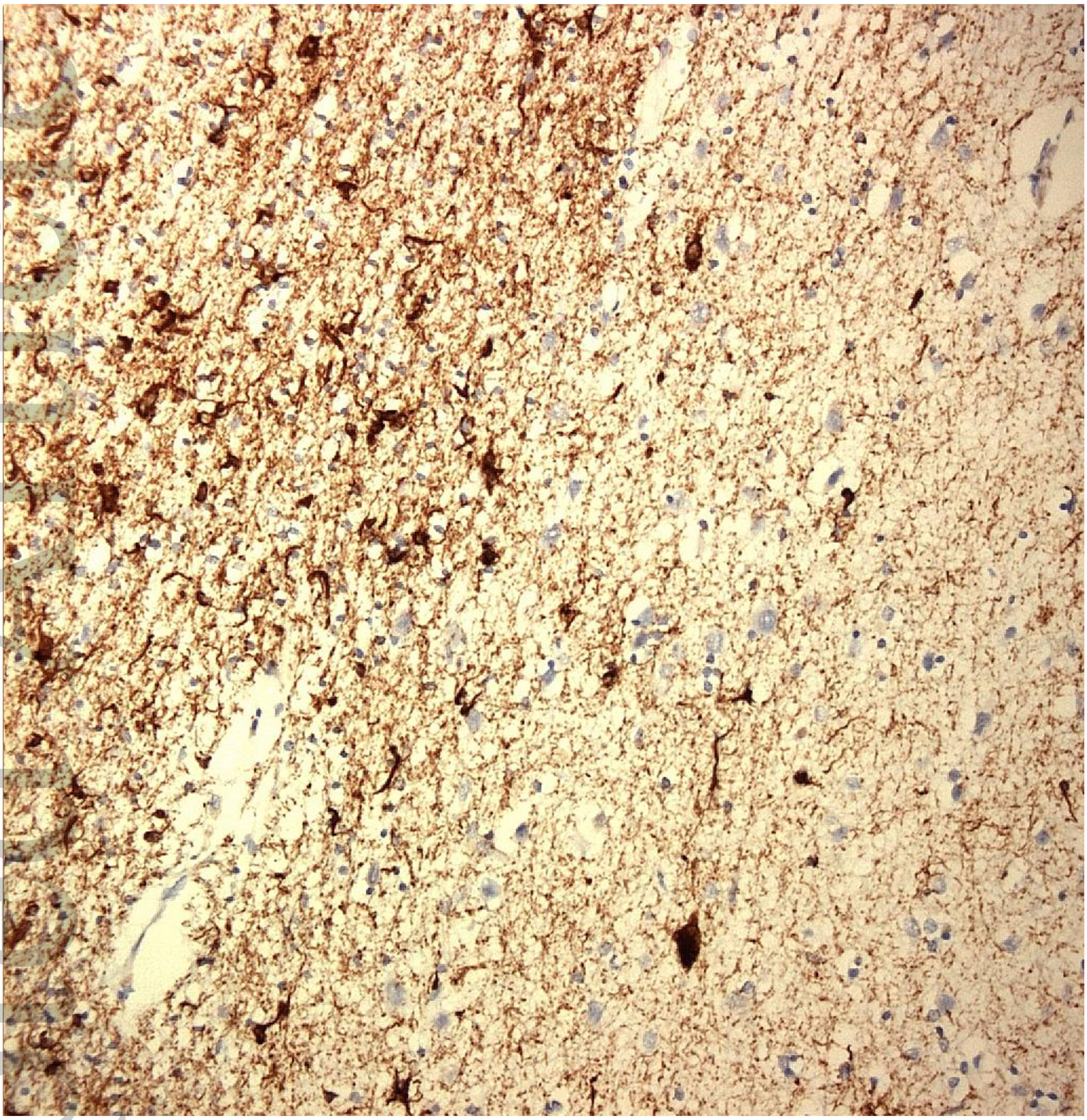
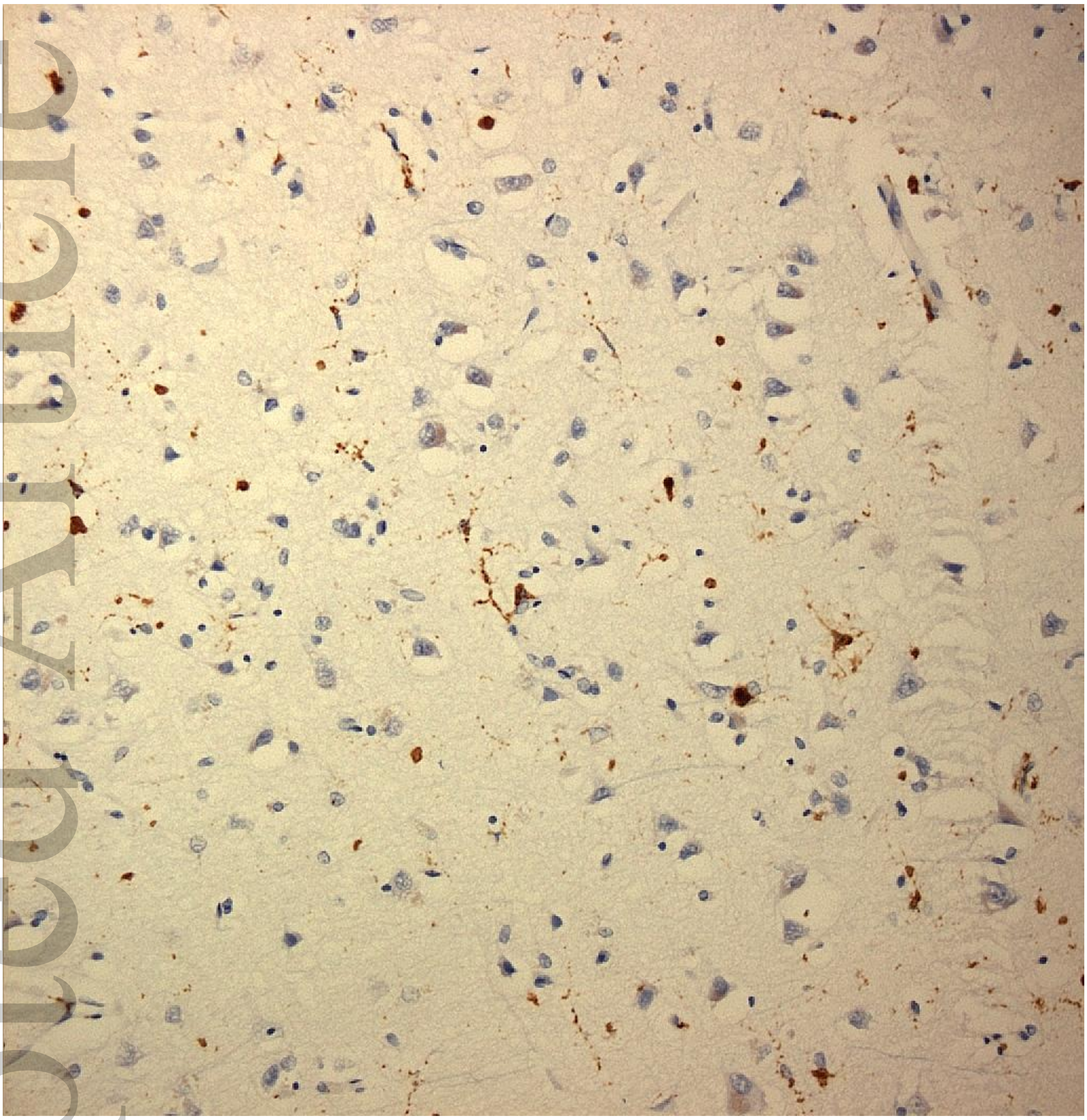
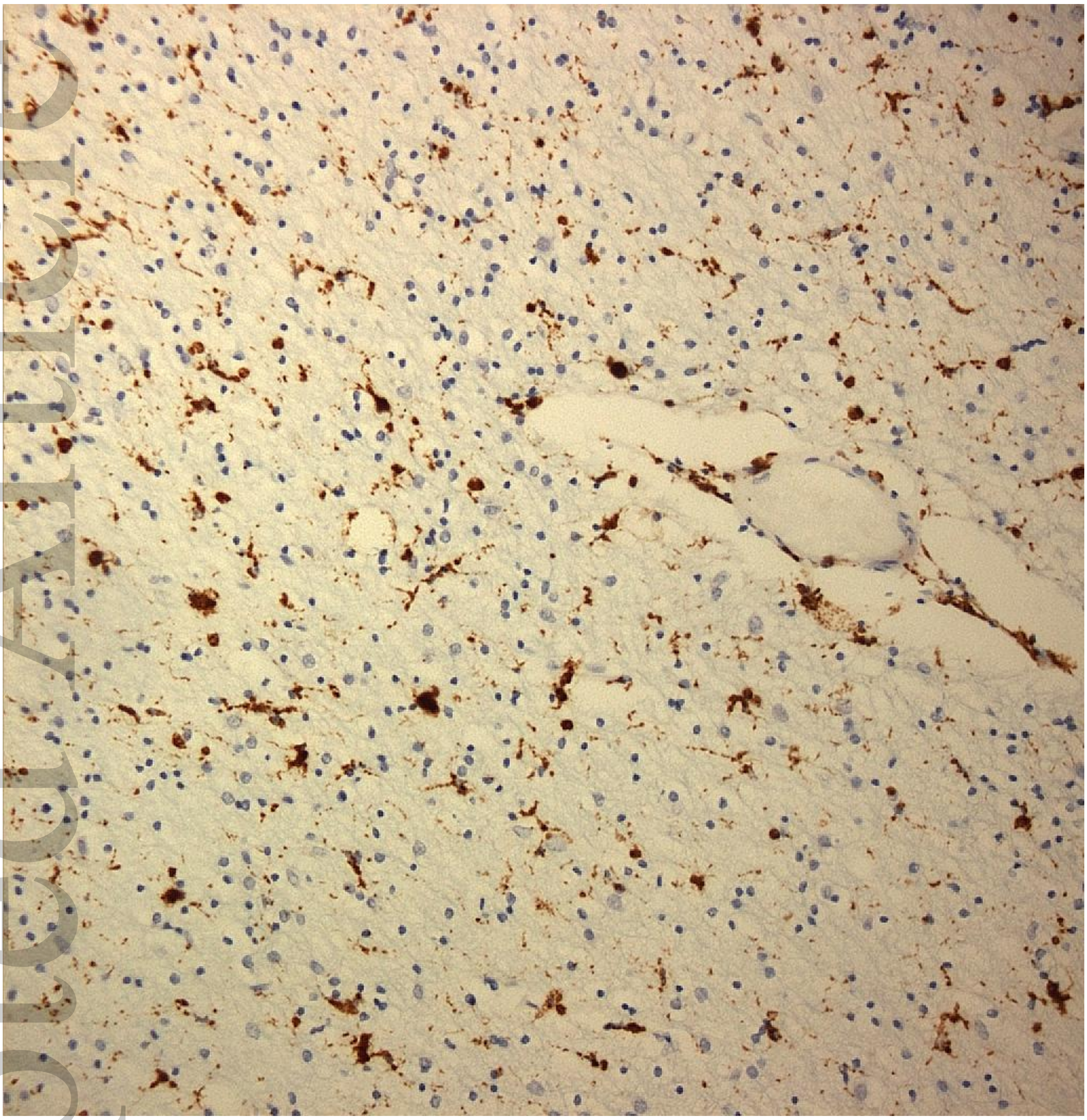


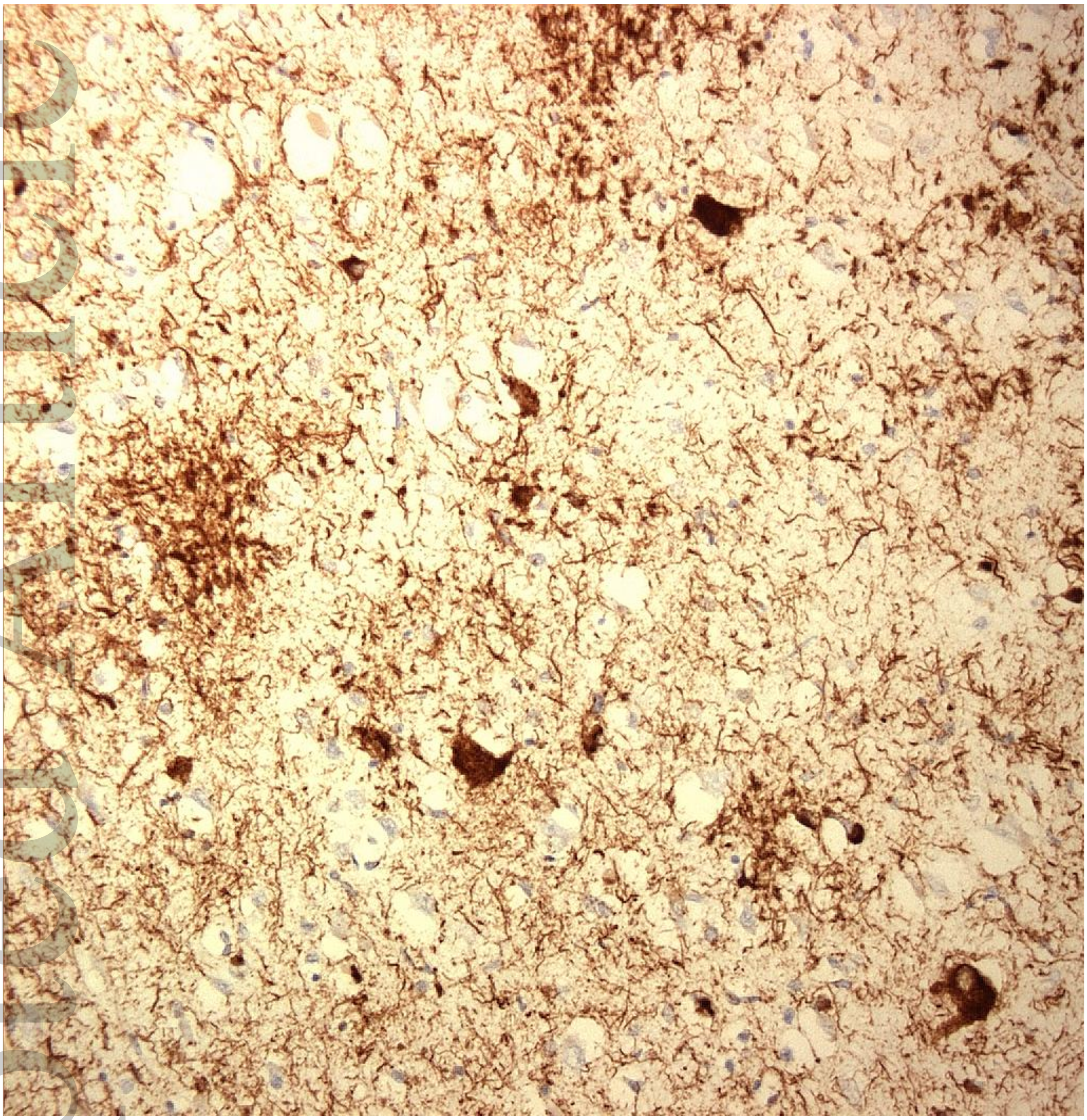
Figure 2. Microglial cells in grey (a) and white (b) matter in FTLD-tau with Pick bodies. Microglial cells are often clustered in outer and deeper (a) cortical layers where Pick bodies are most frequent (c). In FTLD-tau with exon 10 +16 *MAPT* mutation, microglial cells are denser in white (d) than grey (e) matter, in line with greater involvement of oligodendroglial cells in white matter by tauopathy, than of neurones and glia in grey matter (f).

CD68 immunoperoxidase (a,b,d,e), x40 microscope magnification.

AT8 (tau) immunostaining (c,f), x20 microscope magnification.







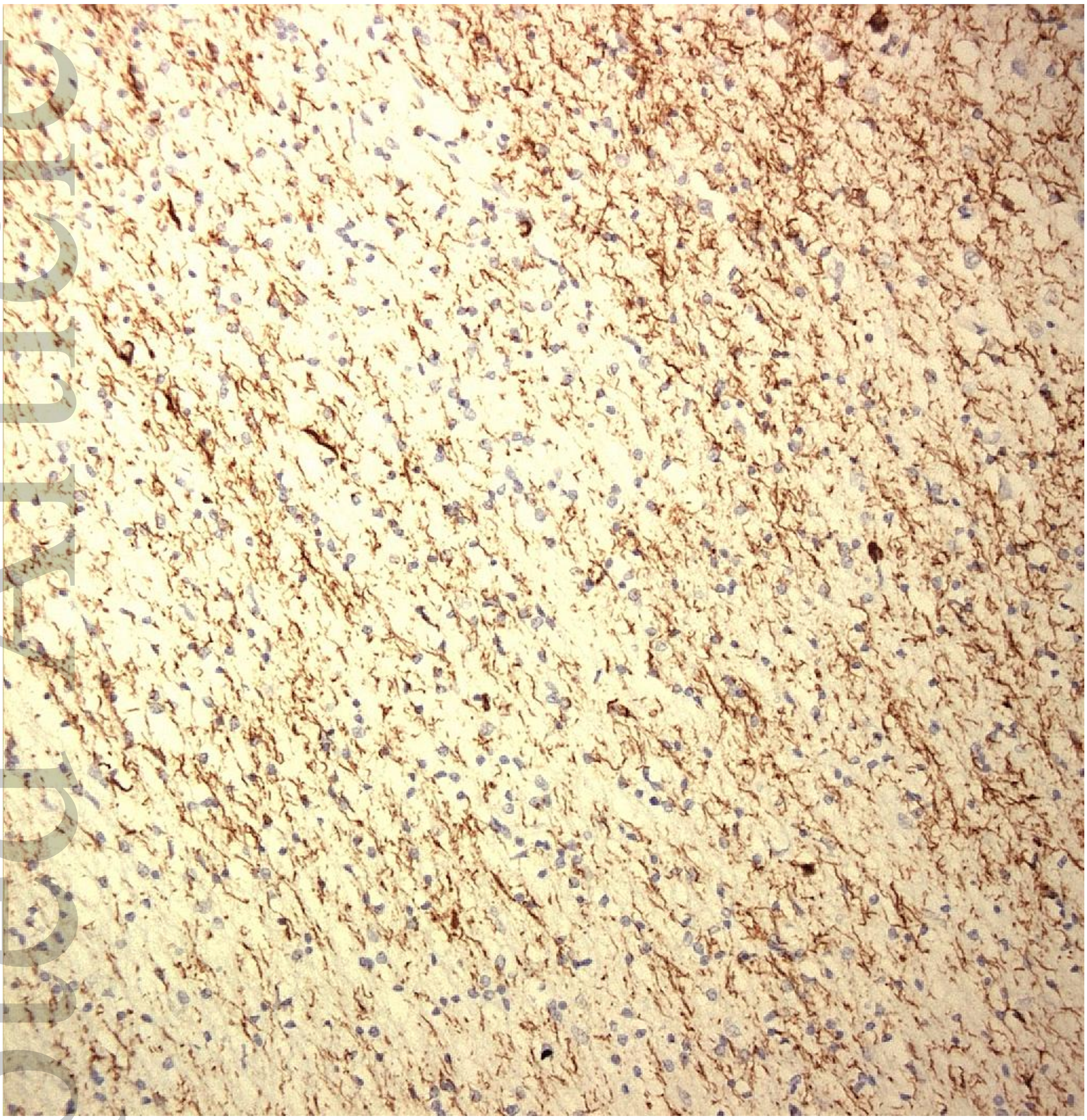


Figure 3. Microglial cells in grey (a) and white (b) matter in CBD. There appears to be more microglial cells in grey than white matter, in line with the heavy involvement of tau positive astrocytes in grey matter (as astrocytic plaques) (c) and white matter as coiled bodies (d).

CD68 immunoperoxidase (a,b), x40 microscope magnification.

AT8 (tau) immunostaining (c,d), x20 microscope magnification.

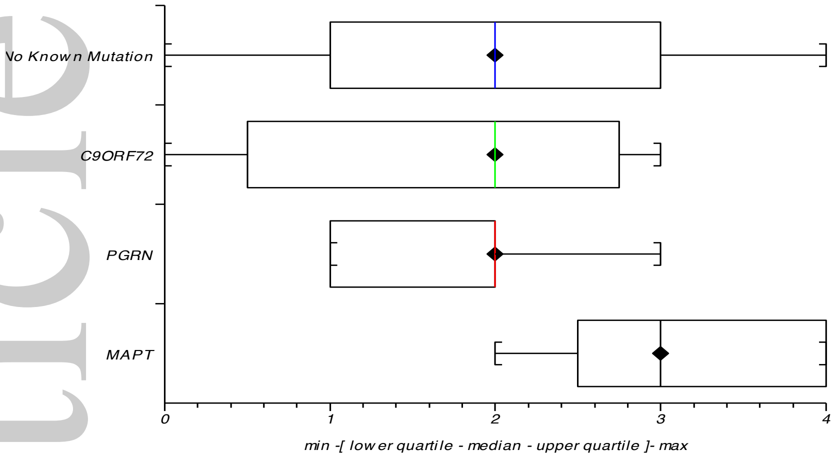


Figure 4. Extent of microglial activity in the subcortical white matter of the temporal cortex in cases with known FTLD-associated genetic mutations (*MAPT*, *GRN*, *C9ORF72*) compared those without known mutations.

On line Supplementary Table 1. Case details